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Methionine-101 from one strain of H5N1 NS1 protein determines its IFN-antagonizing ability and subcellular distribution pattern

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Influenza A virus NS1 protein has developed two main IFN-antagonizing mechanisms by inhibiting retinoic-acid-inducible gene I (RIG-I) signal transduction, or by suppressing cellular pre-mRNA processing through binding to cleavage and polyadenylation specific factor 30 (CPSF30). However, the precise effects of NS1 on suppressing type I IFN induction have not been well characterized. Here we report that compared with PR/8/34 NS1, which is localized partially in the cytoplasm and has strong IFN-antagonizing ability via specifically inhibiting IFN-β promoter activity, H5N1 NS1 has strikingly different characteristics. It mainly accumulates in the nucleus of transfected cells and exerts rather weak IFN-counteracting ability through suppression of the overall gene expression. The M101I mutation of H5N1 NS1, namely H5-M101I, fully reversed its functions. H5-M101I gained the ability to specifically inhibit IFN-β promoter activity, translocate to the cytoplasm, and release CPSF30. The previously reported NES (nuclear export signal) (residues 138−147) was unable to lead H5N1 NS1 to translocate. This suggests that other residues may serve as a potent NES. Findings indicated that together with leucine-100, methionine-101 enhanced the regional NES. In addition, methionine-101 was the key residue for the NS1-CPSF30 interaction. This study reveals the importance of methionine-101 in the influenza A virus life cycle and may provide valuable information for antiviral strategies.

influenza A virus, H5N1, NS1, IFN-β, CPSF30, NES

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Influenza A virus, which belongs to the Orthomyxoviridae family, is an enveloped, single-stranded RNA virus. Its genome is made up of eight negative RNA segments, among which, segment 8 encodes one of the two non-structural proteins [1–3], non-structural protein 1 (NS1). NS1 has two functional domains, the N-terminal 73-amino-acid RNA-binding domain (RBD) and the C-terminal effector domain (ED) [4]. As a viral encoded virulence factor [5–7], NS1 protein has developed two main IFN-antagonizing mechanisms. It sequesters dsRNA [8–10] generated during virus infection and inhibits TRIM25-mediated activation of RIG-I [11,12], thereby blocking the type I interferon signaling pathway. In addition, certain NS1 proteins have evolved another way to bind to cleavage and polyadenylation specific factor 30 (CPSF30) [13,14], a subunit of CPSF [15] and an essential component of the 3′ end processing machinery of cellular pre-mRNAs [15–17], hence, inhibiting cellular pre-mRNA processing, including IFN-β pre-mRNA [14,18].

In 1998, a latent nuclear export signal (NES) of NS1 protein was determined. This NES is localized in the N-terminus of the ED (residues 138 to 147), and is only present in transfected but not virus-infected cells. NS1 protein accumulates in the nucleus since the nuclear exporting function is inhibited by the adjacent amino acid sequence [19].

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In the present study, we revealed dramatic differences between the NS1 proteins from PR/8/34 and H5N1 in both IFN-antagonizing ability and subcellular localization patterns. NS1 from PR/8/34 does not bind to CPSF30 as indicated by others [20–23]. Furthermore, in our study, H5N1 NS1 was localized in the nucleus in transfected cells and had weak IFN-counteracting ability via binding to CPSF30. By construction of a series of mutants and chimeras based on H5N1 NS1, we found that a single amino acid substitution of methionine-101 with isoleucine (H5-M101I), although not even close to the previously reported NES (residues 138 to 147), altered both the subcellular distribution pattern as well as the IFN-antagonizing ability.

As H5N1 infection is a zoonotic disease, the predominant pathway of human infection is still avian-to-human transmission. However, the human infection is still sporadic and rare [24]. Our study may help in the control of human infection of H5N1.

1 Materials and methods

1.1 Cells and plasmids

293T and HeLa cells were maintained in DMEM supplemented with 10% FBS (Invitrogen) at 37°C with 5% CO_2. Expression constructs for NS1 under the control of chicken β-actin promoter (pCAGGS-NS1) were created using *Eco*R I and *Xho* I with an N-terminally tagged HA. Chimera pCAGGS-HA-5N8C was constructed to express an HA-NS1 fusion protein with amino acids 1-73 (RBD) from H5N1 NS1 and 74-230 (ED) from influenza A/PR/8/34 (PR8). Chimera pCAGGS-HA-8N5C was constructed to express RBD from PR/8/34 and ED from H5N1 NS1. The last 17 residues from the C-terminus of H5N1 NS1 were substituted for the corresponding amino acids from PR/8/34 NS1 to construct H5-C17. H5-F98S and H5-M101I were created with a F98S and a M101I single mutation, respectively. H5-98/101 was a mutant based on H5N1 NS1 with F98SM101I double mutations. H5-98-113 contains F98SM101IS109PK113R quadruple mutations. TMASV, five residues from PR/8/34 NS1 80-84aa, were added to the corresponding sites of the H5N1 NS1 to generate H5-TMASV. H5-TM-98-113 was created by overlapping PCR using H5-TMASV as a template. Position 70 was changed from E to K by overlapping PCR to generate H5-E70K-TMASV. Human CPSF30 was cloned into pCAGGS by *Sma* I and *Xho* I using the human cDNA clone CMV6-CPSF30 (Origene) as a template. Reporter plasmid p125-Luc (IFN-β-Luc) was described previously [25]. pRL-TK was purchased from Promega and pEF-flag-RIG-I was kindly provided by Dr. T. Fujita.

1.2 Reporter gene assays

For activation of the IFN-β promoter, 293T cells in 24-well plates were co-transfected with 25 ng internal control plasmid pRL-TK, 125 ng reporter plasmid p125-Luc, and 500 ng of the indicated NS1 expression plasmids or 500 ng empty vector pCAGGS using the Calcium Phosphate-mediated transfection method of the Mammalian Transfection kit (Promega). Twenty-four hours post-transfection, cells were mock infected or infected with SeV for 16 h. Cells were lysed by 1× Passive Lysis Buffer at room temperature for 15 min. Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega).

1.3 Western blot analysis

Cells were washed with PBS once and lysed by lysis buffer for Western and Immunoprecipitation (Beyotime) in the presence of Protease Inhibitor Cocktail (Roche). Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidenedifluoride (PVDF) membranes (Millipore). NS1 proteins were detected by polyclonal rabbit anti-NS1 or rabbit anti-HA (Ab-mart). Monoclonal anti-β-actin antibody was purchased from Beyotime and Monoclonal antibody against Flag was purchased from Sigma.

1.4 Immunofluorescence and confocal microscopy

To detect the subcellular location of NS1, HeLa cells grown on glass slides were transfected with NS1-expression plasmids. Twenty-four hours post-transfection, cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton-X 100 for another 15 min at RT, followed by blocking with 5% BSA overnight at 4°C. After washing once with PBS, the cells were incubated with anti-HA antibody (Sigma-Aldrich) at 4°C overnight. Cells were then washed three times, and incubated for 40 min with the secondary antibody, either fluorescein isothiocyanate-conjugated (FITC) or rhodamine-conjugated antibody (Pierce). After four washes with PBS, cells were stained with Hoechst 33258 (Beyotime). Cells were incubated at room temperature for 10 min, washed three times for 5 min each, and examined by TCS SP2 laser scanning confocal microscope (Leica, Wetzlar, Germany).

1.5 Coimmunoprecipitation (CoIP)

293T cells in 6-well plates were transfected with 2 μg NS1 expression plasmids or pCAGGS and 2 μg pCAGGS-CPSF30-flag separately. Twenty-four hours post-transfection, cells were washed with cold PBS once, lysed in lysis buffer, and clarified by centrifugation at 16000×*g* for 5 min at 4°C. CoIP assays were performed using Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) according to manufacturer's recommendations. Briefly, 10 μg anti-Flag mAb M2 (Sigma) or nonspecific mouse IgG diluted in 200 μL Ab Binding & Washing Buffer were incubated with

50 μL Dynabeads for 10 min at room temperature to allow formation of a Dynabeads-Ab complex. After gently washing, the beads were incubated with cell lysates for 10 min to allow Ag to bind to the Dynabeads-Ab complex. After rinsing four times, the captured Ags were eluted and subjected to SDS-PAGE and Western blot analysis.

2 Results

2.1 H5N1 NS1 protein had relatively weak IFN-antagonizing ability compared with that of PR/8/34 NS1

Influenza A virus NS1 protein is a virulence factor in counteracting IFN-mediated antiviral responses of the host [5–7]. It was previously reported that NS1 differed markedly in inhibiting IRF3 activation and IFN-β transcription [26]. In the present study, we found that unlike PR/8/34 NS1, NS1 of H5N1 differed significantly in its ability to inhibit IFN-β promoter activation and affect expression of reference genes (Figure 1A). As reported previously, PR/8/34 NS1 could enhance reporter gene expression when co-transfected with 293T cells [27]. Similarly, we found that reference gene (RL-TK) expression level in PR/8/34 NS1-transfected cells increased four times compared with that in empty vector (pCAGGS)-transfected cells, whilst the *firefly* luciferase (IFN-β-Luc) gene expression was reduced by around 20 times. In terms of the H5N1 NS1 protein, RL-TK and IFN-β-Luc expression levels were both decreased. However, the former was reduced to a much greater extent, which resulted in a significantly higher relative luciferase activity compared with that of PR/8/34 NS1. We concluded that both PR/8/34 and H5N1 NS1 could suppress IFN-β promoter activity. The former severely blocked the IFN-β signaling pathway. On the other hand, the latter moderately blocked the IFN production by generally inhibiting host gene expression. Since the expression level of PR/8/34 NS1 was significantly higher than that of H5N1 NS1, we transfected 293T cells with decreasing amounts of PR/8/34 NS1 expression plasmid in reporter gene assays to test whether the lower expression level was the main cause of the weak IFN-antagonizing ability in the case of H5N1 NS1. Figure 2A shows that even at the same expression level, PR/8/34 still had a much stronger ability to suppress IFN-β promoter activity than that of H5N1.

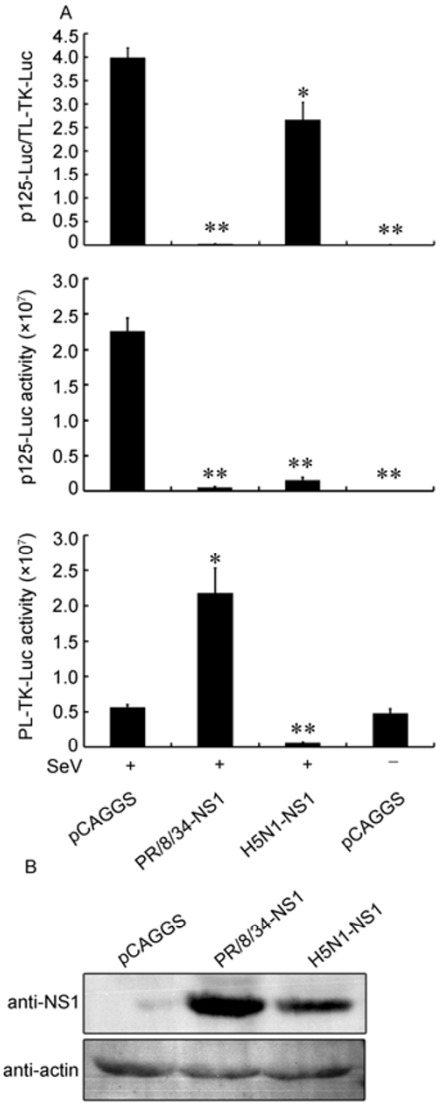

Figure 1 H5N1 NS1 protein has relatively weak IFN-antagonizing ability compared with that of PR/8/34 NS1. A, 293T cells were transfected with 500 ng NS1 expression plasmids or pCAGGS together with 50 ng pRL-TK and 250 ng p125-Luc. 24 h post-transfection, cells were left mock infected or infected with SeV. 16 h later, total cell lysates were collected and subjected to luciferase reporter gene assays. pCAGGS transfected cells with or without SeV treatment were used as positive and negative controls, respectively. B, The corresponding protein expression levels were analyzed by Western blot. Data are representative of at least two independent experiments, with each determination performed in duplicate (mean±SD). *, $P<0.05$; **, $P<0.01$, as determined by student's t test. Immunoblotting of β-actin was used to verify equal loading of protein in each lane.

2.2 The effector domain of H5N1 NS1 protein determined its IFN-antagonizing ability and subcellular distribution pattern

Influenza A virus NS1 protein has two domains, N-terminal RNA-binding domain (RBD, amino acids 1–73) and C-terminal effector domain (ED, residue 74 to the last C-terminal residue). To determine which domain contributes more to counteracting IFN, two chimeras were constructed with an N-terminal-tagged HA, named pCAGGS-HA-5N8C (an HA-NS1 fusion protein with amino acids 1–73 (RBD) from H5N1 NS1 and 74–230 (ED) from influenza A/PR/8/34 (PR8)) and pCAGGS-HA-8N5C (RBD from PR/8/34 and ED from H5N1 NS1), respectively. We subsequently analyzed their ability to inhibit IFN-β promoter activity. As shown in Figure 3A, chimera 5N8C but not 8N5C strongly suppressed the IFN-β promoter activity, suggesting that the ED of H5N1 NS1 might play a dominant

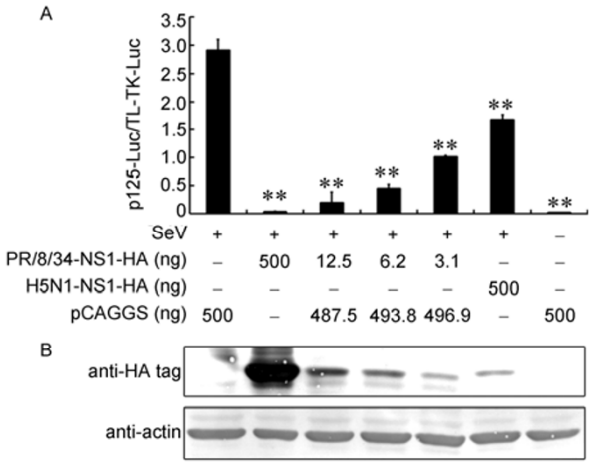

Figure 2 Even at the same expression level, PR/8/34 still possessed a much stronger ability to suppress IFN-β promoter activity compared with that of H5N1. A, 293T cells were transfected with the indicated amount of PR/8/34 NS1 (lanes 2–5), 500 ng H5N1 NS1 (lane 6) or 500 ng pCAGGS (lanes 1 and 7) together with 50 ng pRL-TK and 250 ng p125-Luc. 24 h post-transfection, cells were mock infected or infected with SeV. 16 h later, total cell lysates were collected and subjected to luciferase reporter gene assays. pCAGGS transfected cells with or without SeV treatment were used as positive and negative controls, respectively. B, The corresponding protein expression levels were analyzed by Western blot. Data are representative of at least two independent experiments, with each determination performed in duplicate (mean±SD). *, $P<0.05$; **, $P<0.01$, as determined by student's t test. Immunoblotting of β-actin was used to verify equal loading of protein in each lane.

role in its IFN-antagonizing ability. What is more, chimera 5N8C was similar in its ability to inhibit the reference gene and 125-Luc reporter gene expression. Given that PR/8/34 NS1 is partially targeted to the cytoplasm and remarkably inhibited IFN-β promoter activity, we performed confocal microscopy assays to confirm if chimera 5N8C could translocate. As shown in Figure 4, both the PR/8/34 and chimera 5N8C NS1 proteins partially translocated to the cytoplasm. We hypothesize that the subcellular distribution pattern of NS1 protein might correlate with its IFN-counteracting capability.

2.3 NS1 with M101I mutation translocated to the cytoplasm with enhanced IFN-antagonizing ability

To further test our hypothesis above, we analyzed the NLS (nuclear location signal) and NES of H5N1 NS1. In influenza A virus NS1 protein, NLS2 is within the ED (effector domain) between amino acids 203 to 237, with the basic residues 219, 220, 224, 229, 231 and 232 being most critical, and was confirmed to be still functional even though the strain-specific C-terminal extension (residues 231 to 237) was deleted [28,29]. We aligned the sequences of the C-terminal 31 amino acids between PR/8/34 and H5N1 NS1 by DNAStarMegAlign program and noticed that there was a five-residue difference within the last 17 amino acids (data not shown). Therefore, the corresponding sites of H5N1

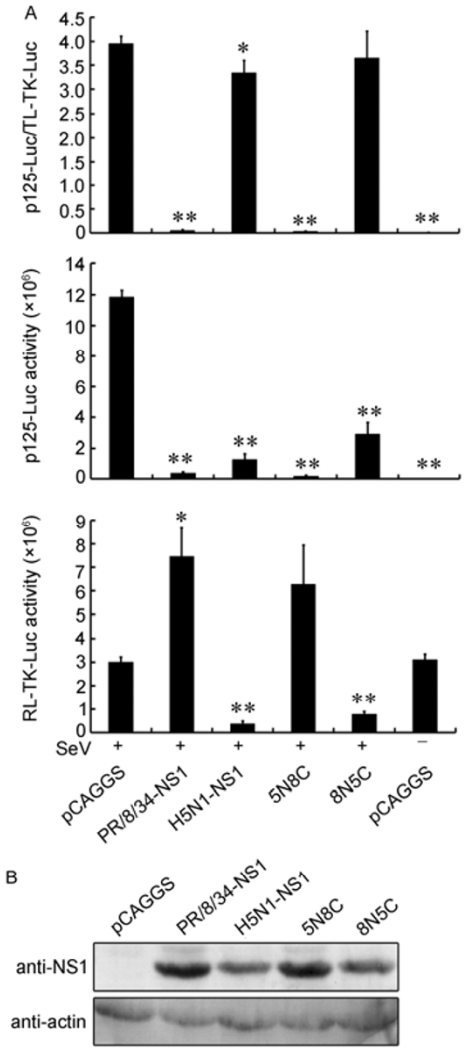

Figure 3 The effector domain of H5N1 NS1 protein determines its IFN-antagonizing ability. A, 293T cells were transfected with 500 ng NS1 expression plasmids (PR/8/34, H5N1, chimera 5N8C containing H5N1 RBD and PR/8/34 ED, chimera 8N5C containing PR/8/34 RBD and H5N1 ED, or pCAGGS together with 50 ng pRL-TK and 250 ng p125-Luc. 24 h post-transfection, cells were mock infected or infected with SeV. 16 h later, cell lysates were subjected to luciferase reporter gene assays. pCAGGS transfected cells with or without SeV treatment were used as positive and negative controls, respectively. B, The corresponding protein expression levels were analyzed by Western blot. Data are representative of at least two independent experiments, with each determination performed in duplicate (mean±SD). *, $P<0.05$; **, $P<0.01$, as determined by student's t test. Immunoblotting of β-actin was used to verify equal loading of protein in each lane.

were substituted with those from PR/8/34 to create H5-C17. Reporter gene assays showed 2–3 fold enhancement in the ability of H5-C17 to inhibit IFN-β promoter activity (data not shown). We further analyzed its subcellular distribution pattern by immunofluorescence and from 12 to 48 h post-transfection, no detectable cytoplasmic NS1 was observed (data not shown). By predicting NES of H5N1 NS1 using NetNES 1.1 (http://www.cbs.dtu.dk/services/NetNES), we noticed that the single mutation E70K plus

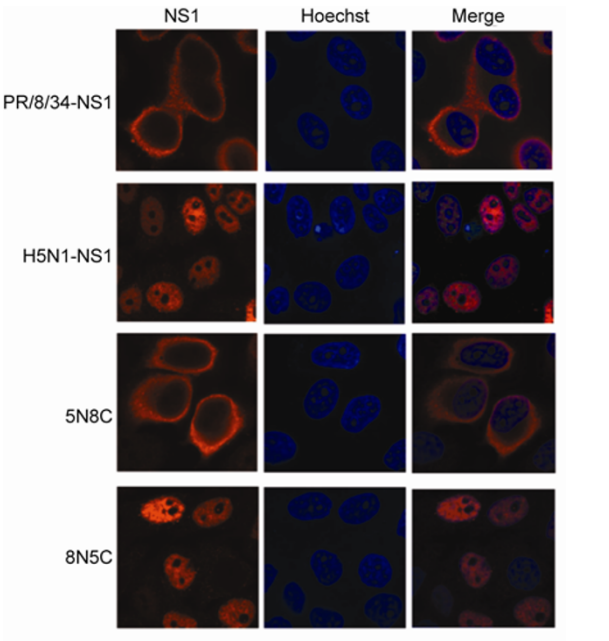

Figure 4 The effector domain of H5N1 NS1 protein determines its subcellular distribution pattern. HeLa cells were transfected with 700 ng NS1 expression plasmids (PR/8/34, H5N1, 5N8C and 8N5C). 24 h post-transfection, cells were fixed and analyzed by immunofluorescence using monoclonal antibody against HA tag.

addition of TMASV to the corresponding site of H5N1 restored an NES signal, which was similar to that of PR/8/34. However, this NES did not lead to the translocation of NS1 12–48 h post-transfection, and did not enhance the IFN-antagonizing ability (data not shown). Next, we created a series of mutants based on H5N1 NS1 at the N-terminus of the effector domain (ED), from residue 79 to 113. We found that the M101I single mutation (H5-M101I) was sufficient to render the ability of NS1 to translocate from the nucleus to the cytoplasm (Figure 5). H5-M101I, as expected, also enhanced reference gene expression greatly and dramatically inhibited IFN-β promoter activity specifically (Figure 6A). These data suggest that methonine-101 of H5N1 NS1 protein determines both the intracellular localization pattern, IFN-antagonizing capacity and probably even the mechanism.

2.4 H5-M101I weakly interacted with CPSF30

Methionine 101 from H5N1 NS1 is one of two residues that play an important role in stabilizing the CPSF30-NS1 complex [20–23,25]. We proposed that the 101 mutant might lose the ability to interact with CPSF30. To test this hypothesis, coimmunoprecipitation (CoIP) was performed (Figure 7). Previous studies indicated that PR/8/34 NS1 had little or no binding ability to CPSF30 [20–22,25]. As shown in Figure 7, there was also no detectable binding between PR/8/34 NS1 and CPSF30. In addition, H5-M101I significantly lost its ability to interact with CPSF30.

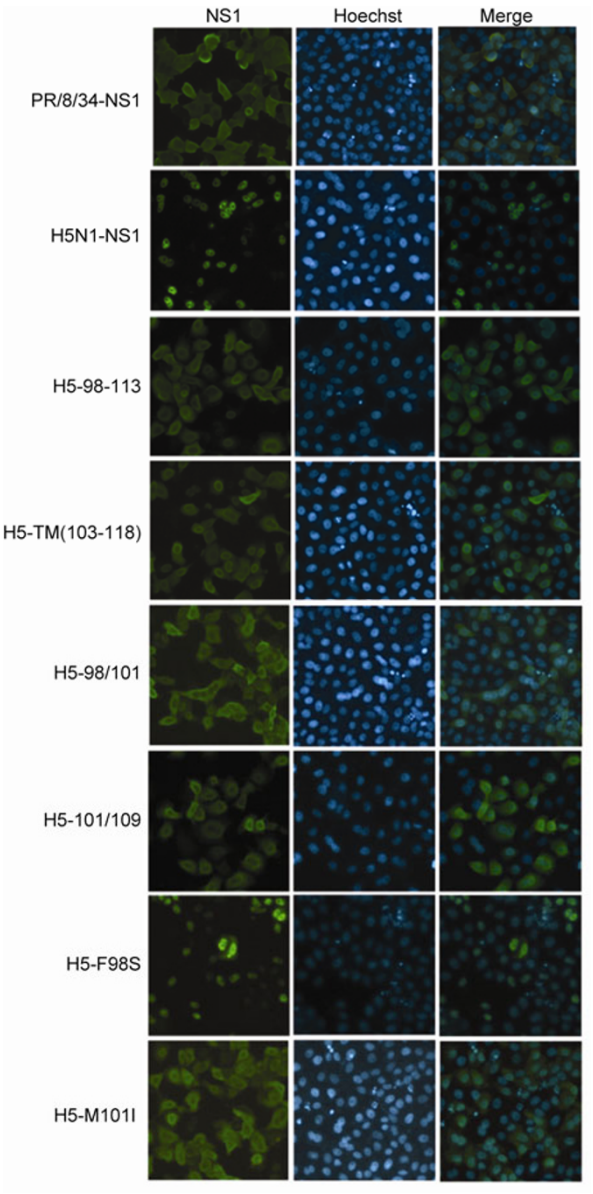

Figure 5 H5-M101I single mutation is sufficient to render the ability of NS1 protein to translocate to the cytoplasm. HeLa cells were transfected with the indicated NS1 expression plasmids. 24 h post-transfection, cells were fixed and subjected to immunofluorescence assays using monoclonal antibodies against HA.H5-98/101, H5N1 NS1 with F98SM101I double mutations. H5-F98S, H5N1 NS1 with F98S single mutation. H5-M101I, H5N1 NS1 with M101I single mutation. H5-98-113, H5N1 NS1 with F98SM101I S109PK113R quadruple mutations. H5-TMASV-103-118, residues 80 to 84 (TMASV) from PR/8/34 NS1 were added to the corresponding sites of H5-98-113. Data are representative of at least two independent experiments.

3 Discussion

In the present study, we further confirmed that one amino acid residue, methionine-101, which is outside the previously reported NES of influenza A virus NS1 protein [19,30] is actively involved in the subcellular distribution process of NS1. Compared with the wild type protein, H5N1 with me-

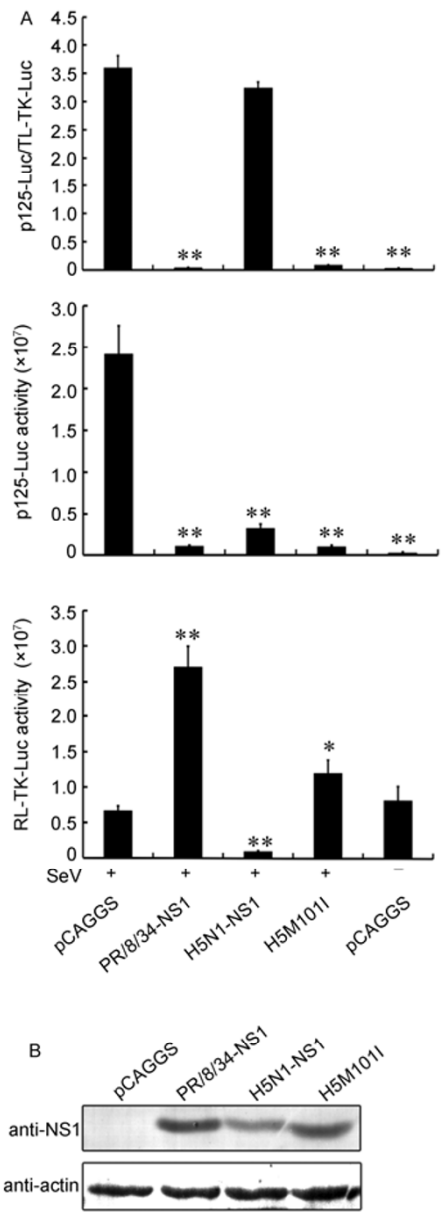

Figure 6 H5-M101I increased its ability in inhibiting IFN-β promoter activity. A, 293T cells were transfected with 500 ng of the NS1 expression plasmids or pCAGGS together with 50 ng pRL-TK and 250 ng p125-Luc. 24 h post-transfection, cells were mock infected or infected with SeV. 16 h later, cell lysates were collected and subjected to luciferase reporter gene assays. pCAGGS transfected cells with or without SeV treatment were used as positive and negative controls, respectively. B, The corresponding protein expression levels were analyzed by Western blot. Data are representative of at least two independent experiments, with each determination performed in duplicate (mean±SD). *, $P<0.05$; **, $P<0.01$, as determined by student's t test. Immunoblotting of β-actin was used to verify equal loading of protein in each lane.

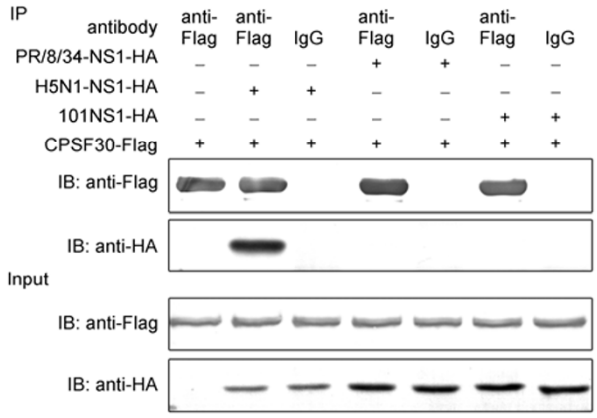

Figure 7 H5-M101I substantially lost the ability to interact with CPSF30. 293T cells were transfected with 2 μg CPSF30 or 2 μg of the indicated NS1 expression plasmids, separately. 24 h later, cell lysates were subjected to coimmunoprecipitation (CoIP) assays. Mouse IgG was used as a negative control.

thionine-101 replaced by isoleucine (H5-M101I) yielded NS1 with a much stronger IFN-antagonizing ability, a change in intracellular distribution, and a different target protein reservoir in the type I IFN signaling pathway. Our findings suggest that by targeting different regions within the cell, NS1 possesses distinct IFN-antagonizing capabilities.

NES is a short amino acid sequence with several critical hydrophobic residues, normally leucines, which are usually spaced, among one another, by a couple of other amino acids [31,32]. It has been reported that isoleucine, another hydrophobic residue, is also critical for the function of NES [33,34]. In H5N1 NS1, there is a leucine (L-100) to the left of methionine-101, implicating that after methionine was substituted by isoleucine (H5-M101I), these two adjacent hydrophobic residues together enhanced the regional nuclear export signal.

Methionine-101 is one of two residues that function to stabilize the CPSF30-NS1 complex [20–23,25]. Consistent with previous reports, the H5-M101I mutant dramatically reduced the ability of NS1 to bind to CPSF30. Recently, another group also revealed that residues responsible for the interaction between NS1 and CPSF30 were related to the localization of NS1 protein in transfected cells [35]. While beyond the scope of this study, it raises an interesting question as to whether the release of CPSF30 is the direct cause of H5-M101I translocation. Based on our findings, it is reasonable to assume that H5-M101I likely gains the ability to interact or dissociate with other cellular proteins which, in turn, may fundamentally change the IFN-antagonizing mechanisms.

The formation of the H5N1-NS1-CPSF30 complex inhibits pre-mRNA processing which affects subsequent protein synthesis in transfected cells. This inhibition was confirmed by the significantly lower *Renilla* luciferase activity observed in reporter gene assays. Although the *firefly* luciferase (125-Luc) activity was reduced significantly, it was not to the same extent as *Renilla* luciferase. There might be a possibility that the remaining CPSF30, which was unbound to H5N1 NS1, tended to show relatively higher affinity to IFN-β than other promoter sequences. Therefore, some mature IFN-β mRNA could still be produced.

Methionine-101 (or 106 for certain NS1) is responsible

for the growth and virulence of influenza A viruses. In recent years, more and more influenza A viruses isolated from humans and avian species have NS1 which encodes methionine at position 101 or 106 [22]. These revealed the crucial role that methionine-101/106 plays in the virus-host battle, although the selective pressure and the mechanisms involved in the virus being able to combat the host immune system remain to be clarified. Apart from the correlation of position 101 and IFN-counteracting ability, our results demonstrated this crucial residue could also influence the subcellular localization of NS1 protein. By targeting different regions within the cell, NS1 might possibly recruit different proteins to combat the host immune system, which contributes to our understanding of virus-host infectivity.

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